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(71) Applicant and

(72) Inventor: **SACKTOR, Todd, C.** [US/US]; 7 Park Hill
Place, Yonkers, NY 10705 (US).

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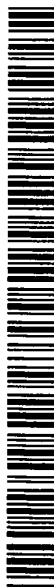
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(74) Agent: **GROLZ, Edward, W.**; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).

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(54) Title: A MEMORY ENHANCING PROTEIN

(57) Abstract: The present invention provides methods and compositions for enhancing memory in animals, including humans by the administration of an effective amount of an atypical form of protein kinase C such as protein kinase M zeta (PKM ζ) or protein kinase C iota/lambda.

BEST AVAILABLE COPY**A MEMORY ENHANCING PROTEIN**

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FIELD OF THE INVENTION

The present invention provides methods for enhancing memory in animals, including humans. The present invention is also directed to methods for treating brain or spinal injury with the administration of an effective amount of atypical forms of protein kinase C, including protein kinase M zeta (PKM ζ). The present invention further provides a method of inducing amnesia with the administration of an effective amount of a PKM ζ inhibitor.

BACKGROUND OF THE INVENTION

A common working hypothesis for the physiological basis of memory is that persistent changes in behavior are mediated by long-term modifications in the strength of synapses (Kandel et al. (1982) Science 218:433-443; Bliss et al. (1993) Nature 361: 31-39). The molecular mechanisms for these changes are complex, involving many signal transduction pathways. Overall, however, these mechanisms are divided into two functionally distinct phases: induction, which initiates the long-term modifications, and maintenance, which sustains the changes (Malinow et al (1988) Nature 335:820-824; Schwartz, J.H. (1993) PNAS 90:8310-8313; Schwartz et al (1987) Ann. Rev. Neurosci. 10:459-476).

Much of the work to examine these signaling

pathways has come from the study of the response to high-frequency afferent stimulation of synapses that causes a long-term increase in synaptic transmission, long-term potentiation (LTP) (Bliss et al. (1993), *supra.*; Bliss et al. (1973) J. Physiol. 232:331-356; Nicoll et al. (1988) 5 1:97-103). The vast majority of signaling molecules implicated in LTP affect only induction, but not maintenance. The exceptions are agents that inhibit the catalytic domain of protein kinases, specifically protein 10 kinase C (PKC), which are able both to block LTP induction and reverse its maintenance. (Nishizuka, Y (1988) Nature 334:661-665; Schwartz, J.H. (1993) *supra.*; Schwartz et al (1987) *supra.*

These two phases can be distinguished 15 experimentally by the timing of the application of pharmacological agents that inhibit signal transduction pathways. When agents are applied prior to a tetanic afferent stimulation and prevent the formation of long-lasting changes, they block induction. If they are 20 applied after the tetanus-and reverse the potentiation that has been established - they affect maintenance.

Several principles have been proposed to characterize mechanisms that might maintain long-term changes in synaptic transmission. First, protein 25 kinases, such as PKC, which transiently enhance synaptic transmission when second messengers are activated, can extend their action by becoming constitutively active kinases that are independent of second messengers. (Schwartz et al (1987) *supra.*; Klann et al. (1991) J. 30 Biol. Chem. 266:24253-24256)

Second, long-term forms of synaptic plasticity are thought to depend upon new protein synthesis, although the critical, newly synthesized molecules that cause synaptic potentiation have not been identified.

- 5 Stanton et al. (1984) J. Neurosci. 4:3080-3088; Frey et al (1988) Brain Res. 452:57-65; Otani et al (1989) Neurosci. 28: 519-526; Abel et al. (1998) Science 279: 338-341. A similar requirement for new protein synthesis has been observed for long-term memory. Davis et al.
10 (1984) Psychol Bull. 96:518-559; Thompson, R.F. Science 233:941-947; Montarola et al. (1986) Science 234:1249-1254.

- While usually considered properties of separate mechanisms, it has been determined that one isoform of
15 PKC possesses both of these features: it is persistently increased during LTP as a constitutively active enzyme, and it is generated by new protein synthesis. Sacktor et al. (1993) Proc. Natl. Acad. Sci. (USA) 90:8342-8346. This newly described form of PKC is PKM ζ , the independent
20 catalytic domain of the PKC ζ isoform, which, lacking PKC ζ 's autoinhibitory regulatory domain, is autonomously active. Schwartz, J.H. (1993) *supra*; Sacktor et al. (1993) *supra*.

- PKM is usually thought to be produced by
25 limited proteolysis of PKC, separating the enzyme's regulatory and catalytic domains. This may occur early after a high-frequency tetanus. Recent evidence shows, however, that the long-lasting PKM ζ may also be derived from a brain-specific mRNA that encodes only the
30 catalytic domain of ζ . Andrea et al. (1995) Biochem. J.

310:835-843; Powell et al. (1994) Cell Growth Differ.
5:143-149.

PKC is a family of multifunctional protein kinases, first discovered by Nishizuka in 1977. Takai et al. (1977) J. Biol. Chem. 252:7603-7609; Inoue et al. (1977) J. Biol. Chem. 252:7610-7616. PKC consists of two domains separated by a hinge region: an amino-terminal regulatory domain, which contains an autoinhibitory pseudosubstrate sequence and second messenger/lipid binding sites, and a carboxy-terminal catalytic kinase domain. PKC is held in an inactive state in the cytosol by the interaction between the regulatory and catalytic domains. When there is an increase in lipid second messengers (or, for some isoforms, Ca^{2+}), PKC translocates from the cytosolic to membranous (or cytoskeletal) compartments, and a change in its conformation occurs, displacing the regulatory from the catalytic domain, releasing the autoinhibition, and activating the enzyme. The 10 different forms of PKC are divided into 3 groups: conventional (α , βI , βII , γ), novel (or new, δ , ϵ , η , θ), and atypical (ζ , ι/λ), each of which is activated by a distinct set of second messengers. (PKD or PKC μ is a PKC-related molecule with a catalytic domain closer to CaM-kinase). The conventional PKCs are activated by Ca^{2+} and diacylglycerol (DAG); the novel by DAG, but not Ca^{2+} ; and the atypical by neither DAG or Ca^{2+} , but by alternate lipid-second messengers, including arachidonic acid, phosphoinositide 3-OH kinase products, and ceramide.

A second mechanism for permanently activating PKC, also discovered by Nishizuka, is the cleavage by

calpain or their proteases at the hinge region, to permanently separate the regulatory from the catalytic domains. The independently active kinase domain is called PKM. ("M" stands for Mg^{2+} , although this requirement turned out to be for the Mg^{2+} in Mg^{2+} -ATP). PKM formation results in a persistently active kinase and is not the typical way PKC is activated. It has been found that stable PKM formation occurs endogenously only for a single isoform, ζ , and only in brain. Naik et al. (submitted for publication). Recently, PKM ζ has also been reported in a neuronally differentiated cell line. Oehrlein et al. (1998) Eur. J. Cell. Bio. 77:323-337.- Stable PKM forms for the other isoforms have been observed only in pathological conditions: PKM ϵ in breast cancer tumor cells (Baxter et al. (1992) J. Biol. Chem. 267: 1910-1917) and heart ischemia (Urthaler et al. (1997) Cardiovasc. Res. 35:60-67) and PKM δ in apoptosis (Emoto et al. (1996) Blood 97:1990-1996; Denning et al. (1998) J. Biol Chem. 273:29995-30002).

Protein kinase M zeta (PKM ζ) is a form of protein kinase C which has a fundamental role in the formation and maintenance of memory. PKM ζ is a critical molecule in the most widely-studied physiological model of memory, long-term potentiation (LTP) of synapses (Sacktor, et al., (1993) *supra.*; Osten, et al., (1996) J. Neurosci. 16(8):2444-2451; Hrabetova and Sacktor, (1996) J. Neurosci. 16(17):4324-5333).

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for enhancing memory and treating brain and

spinal cord injury by the administration of a therapeutically effective amount of one or more atypical forms of protein kinase C (PKC) such as PKM ζ or PKC iota/lambda.

5 In one aspect, the present invention provides method of enhancing synaptic transmission in an animal comprising the administration of a therapeutically effective amount of one or more atypical forms of PKC such as PKM ζ or PKC iota/lambda.

10 In another aspect, the present invention provides a method of maintaining memory in an animal comprising the administration of a therapeutically effective amount of one or more atypical forms of PKC such as PKM ζ or PKC iota/lambda.

15 In still another aspect, the present invention provides a method for enhancing synaptic transmission or maintaining memory comprising the administration of DNA encoding the human (or animal) sequence of PKM ζ . In yet another aspect, the present invention provides a method
20 for causing amnesia or decreasing synaptic transmission, comprising the administration of a therapeutically effective amount of a PKM ζ inhibitor. Uses for decreasing synaptic transmission include, for example, the treatment of acute or chronic pain, treatment of drug
25 or alcohol addiction, and treatment of epilepsy.

 In still yet another aspect, the present invention provides a method for causing amnesia or decreasing synaptic transmission, comprising the administration of a therapeutically effect amount of a
30 dominant negative PKM ζ inhibitor, DNA encoding the human (or animal) sequence of dominant negative PKM ζ , or

antisense to PKM ζ .

In another aspect, the present invention provides a method of causing amnesia in an animal comprising the administration of a therapeutically effective amount of a PKM ζ inhibitor or a PKC iota/lambda inhibitor.

In still another aspect, the present invention provides a pharmaceutical composition comprising PKM ζ and a pharmaceutically acceptable carrier.

In yet another aspect, the present invention provides a pharmaceutical composition comprising PKC iota/lambda and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows individual EPSC responses to injected PKM ζ .

Figure 1B shows the time course of EPSC amplitudes.

Figure 2A shows whole cell recording of CA1 pyramidal cells with 3nM PKM ζ . Increases in synaptically evoked AMPA/kainate responses with the introduction of PKM ζ were reversible by chelerythrine (1 μ M).

Figure 2B shows the time course of EPSC amplitudes.

Figure 3 shows a silver stain of purified recombinantly expressed PKM ζ .

Figure 4 shows specificity of inhibition of PKM ζ relative to other protein kinases by chelerythrine. PKCA is PKC alpha phosphorylation of myelin basic protein (MBP). PKCE is PKC epsilon phosphorylation of MBP. PKM is PKM ζ phosphorylation of MBP. CKII is Ca²⁺-calmodulin-dependent protein kinase II phosphorylation of Syntide®

(Calbiochem, San Diego, CA.) peptide substrate.

Figure 5 shows that a myristolated zeta inhibitory pseudosubstrate peptide (1 μ M), reverses LTP maintenance.

5 Figure 6 shows the DNA sequence (derived from cDNA) encoding human PKM ζ .

Figure 7 shows postsynaptic exposure to dominant negative inhibitor PKM ζ -K281W (20 nM) prevents LTP. (A) Silver stain of baculovirus/Sf9-expressed PKM ζ -K281W protein placed into whole-cell recording pipette. PKM ζ -K281W appears as a doublet. (B) Depolarization to -40 mV at 1 and 10 min after diffusion of PKM ζ -K281W shows no obvious effect on synaptic responses mediated by AMPA and NMDA receptors. (C) Upper traces, whole-cell EPSCs pre-and posttetanization, showing no persistent potentiation after exposure to PKM ζ -K281W. Lower traces, simultaneously recorded field potentials show LTP. (D) Time course of whole-cell recording with PKM ζ -K281W. Tetanization shows only PTP. Inset, time course of simultaneous field recordings shows LTP.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for enhancing memory and treating brain and spinal cord injury by the administration of a therapeutically effective amount of one or more atypical forms of PKC. In a preferred embodiment an atypical form of PKC is PKM ζ . In another preferred embodiment an atypical form of PKC is PKC iota/lambda. In accordance with the present invention it has been determined that PKM ζ is both necessary and sufficient for the long-term

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maintenance of LTP. Moreover, it has been determined in accordance with the present teachings that the function of PKM ζ is to store and consolidate memories in the brain.

5 In accordance with the present invention, members of the class of compounds known as atypical forms of PKC such as protein kinase M zeta (PKM ζ) and PKC iota/lambda have been found to maintain or consolidate long term changes in synaptic strength in vertebrates,
10 the mechanism for long term memory. The present invention elucidates PKM ζ 's role in maintaining enhanced synaptic transmission with studies of long-term potentiation (LTP). Conversely, inhibition of PKM ζ may cause amnesia, which may be useful in the treatment of
15 traumatic stress disorders, phobias and acute or chronic pain.

 Other agents that have been proposed to enhance memory are essentially stimulates (like coffee) or agents designed to enhance the induction of long-term
20 potentiation (LTP)-like processes (such as drugs to increase cAMP). PKM ζ is the first molecule whose function is to maintain memories in vertebrates. In accordance with the present invention, when PKM ζ is injected into neurons it persistently enhances synaptic
25 transmission.

 In one embodiment the present invention contemplates a method of treating a brain injury in an animal comprising the administration of a therapeutically effective amount of one or more atypical forms of PKC
30 such as PKM ζ or DNA encoding PKM ζ message. By therapeutically effective amount is meant an amount of an

atypical form of PKC high enough to significantly positively modify the condition to be treated but low enough to avoid serious side effects (at a reasonable benefit/risk ratio), within the scope of sound medical judgment. For example, a therapeutically effective amount of PKM ζ will vary with the particular injury being treated, the age and physical condition of the patient being treated, the severity of the injury, the duration of treatment, the nature of concurrent therapy and the specific PKM ζ employed.

In another embodiment the present invention contemplates a method of treating a spinal cord injury in an animal comprising the administration of a therapeutically effective amount of one or more atypical forms of PKC such as, for example, PKM ζ .

In still another embodiment, the present invention contemplates a method of enhancing synaptic transmission in an animal comprising the administration of a therapeutically effective amount of one or more atypical forms of PKC such as, for example, PKM ζ .

The present invention also contemplates a method of inducing amnesia in an animal by the administration of a therapeutically effective amount of a PKM ζ inhibitor. In preferred embodiments the PKM ζ inhibitor is chelerythrine, myristolated zeta inhibitory pseudosubstrate (MZIP) peptide (myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH), or dominant negative form of PKM ζ such as, for example, PKM ζ -K281W, or antisense to PKM ζ mRNA. MZIP has an IC₅₀ of 10-100nM for PKM ζ and 10,000nM for PKC gamma and therefore is a more specific inhibitor than chelerythrine (see Fig. 5).

Candidates for the induction of selective amnesia contemplated by the present invention are preferably humans having, for example, post-traumatic stress disorders and phobias.

5 The present invention also contemplates a method of reducing synaptic transmission in selective areas of the brain or spinal cord by the administration of a therapeutically effective amount of PKM ζ inhibitor. Candidates for the reduction of synaptic transmission
10 contemplated by the invention are preferably humans having, for example, disorders of pain, drug or alcohol addiction, or excess neuronal activity as in epilepsy.

 Still another embodiment of the present invention contemplates pharmaceutical compositions
15 containing one or more atypical forms of PKC such as, for example, PKM ζ .

 The active ingredients of a pharmaceutical composition containing PKM ζ or a nucleic acid encoding PKM ζ is contemplated to exhibit effective therapeutic
20 activity, for example, in enhancing memory, and treating brain and spinal cord injuries. Thus the active ingredients of the therapeutic compositions containing PKM ζ is administered in therapeutic amounts which depend on the particular disease. For example, final
25 concentrations of PKM ζ in brain or spinal cord to be achieved by administration may be about 1 nanomolar. The dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be
30 proportionally reduced as indicated by the exigencies of the therapeutic situation. Administration of one or more

atypical forms of PKC such as, for example, PKM ζ into the brain or spinal cord may be intracranially or intrathecally, i.e., by intrathecal pump or repository. Depending on the route of administration, the active ingredients which comprise PKM ζ may be required to be coated in a material to protect said ingredients from the action of acids and other natural conditions which may inactivate said ingredients.

For example, PKM ζ may be administered in an adjuvant or in liposomes. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Liposomes include water-in-oil-in-water P40 emulsions as well as conventional liposomes.

Under ordinary conditions of storage and use, the preparations of the present invention contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained,

- for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of
- 5 microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases it will be preferable to include isotonic agents, for example,
- 10 sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.
- 15 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are
- 20 prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable
- 25 solutions, the preferred methods of preparation are vacuum-drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.
- 30 It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form

as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of the active material calculated to produce the desired
5 therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly depending on (a) the unique characteristics of the active material and the particular therapeutic effect
10 to be achieved, and (b) the limitations inherent in the art of compounding such as active material for the treatment of injury in living subjects having a condition in which bodily health is impaired as herein disclosed in detail.

15 The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, result in achieving,
20 for example, about 0.1 to about 10 nanomolar concentrations of PKM ζ in the brain or spinal cord.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic
25 and adsorption delaying agents, and the like. The use of such media agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic
30 compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Administration of an atypical form of PKC, such as PKM ζ may also include altered forms or derivatives of PKM ζ or drugs that enhance its activity, stability, or accessibility to the nervous system. The identification of applicable PKM ζ enhancing drugs are readily tested or screened by examining the effects of drugs or PKM ζ 's phosphorylation in vitro (as measured in Figure 4) or on PKM ζ 's effect on synaptic transmission when injected into neurons as in Figure 2.

Administration of PKM ζ DNA into brain or spinal cord may also be by gene-transfer technology. Such technologies include, but are not limited to, viruses, liposomes, and altered forms or derivatives of DNA or RNA.

Administration of inhibitors of PKM ζ activity include drugs, such as chelerythrine, myristolated zeta inhibitory pseudosubstrate peptide and altered forms of PKM ζ that, through dominant negative effects inhibit endogenous PKM ζ 's activity or effectiveness. Such dominant negative agents include, but are not limited to, inactive forms or portions of PKM ζ . Inhibition of PKM ζ function may also include decreasing levels of endogenous PKM ζ through administration of antisense to the sequence of PKM ζ .

The following Examples serve to further illustrate the invention without in any way limiting same.

EXAMPLE 1

The effect of the increase of PKM ζ on synaptic transmission.

5 Intracellular perfusion of PKM ζ .

PKM ζ , purified from a baculovirus/Sf9 expression system to near homogeneity, was perfused into a CA1 pyramidal cell through a whole cell recording pipette (Fig. 1A-1B). The concentration of PKM ζ was
10 about 0.8 nM PKM ζ , with 0.03 pmol/min/ μ l activity. In order to isolate AMPA/kainate responses, the cell, recorded at the soma, was voltage-clamped at the IPSC reversal potential (\sim -70) and Cs-gluconate-based electrode solution was used (to block GABA $_A$ effects).
15 Stimulation was every 10 sec in the radiatum, and input resistance was monitored and did not change throughout the experiment. Over 5-10 min, there was \sim 60% increase in AMPA/kainate EPSCs, which then stabilized. In contrast, inactivated PKM ζ had no effect. In comparison
20 with the concentration of autoactive thiophosphorylated CaM-kinase II reported to potentiate synaptic responses in CA1 pyramidal cells by similar whole cell techniques, the findings on the potentiation of AMPA/kainate currents indicated that PKM ζ was 200- to 1000-fold more potent
25 than CaM-kinase II.

Electrophysiological recording.

Hippocampal slices were prepared from Sprague-Dawley rats as described in Example 2. Patch electrodes
30 were pulled (two-stage) from 15 mm O.D. borosilicate glass (World Precision Instruments, Sarasota, FL) on a Narishige PP-83 vertical puller. Recording pipettes had

tip resistances of 2-5 M Ω and contain (in mM): Cs-gluconate or K-gluconate 130-; MgCl₂, 2; CaCl₂, 2; EGTA, 10; HEPES, 10; Na-ATP, 2; pH adjusted to 7.25 with either CsoH or KOH. This mixture had been shown to reduce the
5 rundown of GABA_A-receptor mediated responses. Cesium was used to block potassium currents, including slow GABA_B IPSCs. Electrode solutions included QX-314 (10mM) to block Na²⁺ currents to prevent cell spike discharges at depolarized holding potentials. Whole-cell recordings
10 were obtained from CA1 pyramidal cells using blind patch techniques. The recording pipette was slowly advanced through the tissue, with brief voltage steps (-10mV, 10 ms) applied to monitor electrode resistance. Once a deflection in the electrode's current response was
15 detected signifying contact with the membrane of the target cell soma, slight negative pressure was applied to form a cell-electrode seal of >1 G Ω .

Membrane breakthrough was accomplished with either additional suction or current pulses. Following
20 membrane rupture, 2 min of settling time was allowed before formal recording. Voltage steps (-10 mV) were applied to monitor the access resistance and input capacitance. Signals were recorded under voltage-clamp with an Axoclamp 2A amplifier (Axon Instruments, Foster
25 City, CA). Resting input resistance was measured from the current response to a -10 mV voltage step from holding potential (usually resting membrane potential, 60 mV with the intracellular solution). Cells were accepted for study only if resting input resistances of
30 >100 M Ω and access resistances <20 M Ω were observed.

If cell access resistance increased significantly during the course of the recording (>20%),

the data was discarded. Data signals were digitized at 94 kHz via a 14-bit PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Element, NY) and stored on VHS tape for later analysis with pCLAMP software (Axon Instruments) on an IBM-compatible Pentium-II microcomputer.

Synaptic events were evoked by extracellular stimulation with bipolar, coated tungsten electrodes placed in stratum radiatum lateral to the recording electrode. Cathodal shocks (2-10 V; 200 μ s duration) were delivered through a digitally controlled stimulus isolation unit (World Precision Instruments) at a low frequency (0.1 Hz). Unless otherwise noted, drugs were delivered in the bath.

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EXAMPLE 2

Methods: Preparation of hippocampal slices. After anesthesia with halothane, transverse 450 μ m slices were prepared from 3-4 week old Sprague-Dawley rats with a
5 McIlwain tissue chopper. During the dissection, the hippocampus was kept cold with multiple washes of a dissection saline at 4°C (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 11 mM glucose, 10 mM MgCl₂ and 0.5 mM CaCl₂, pH 7.4, equilibrated with 95% O₂/5% CO₂).

10 The slices were transferred to an interface chamber (Fine Science Tools) for incubation in the dissection saline for 1 hour at 32°C. The divalent ion concentrations were then changed to 1.2 mM MgCl₂ and 1.7 mM CaCl₂ (physiological saline).

15 Intracellular perfusion of PKM ζ .

Analysis of the effect of PKM ζ on evoked EPSCs in CA1 pyramidal neurons and effects of chelerythrine.

The effects of PKM ζ cell injection on
20 excitatory synaptic currents were assessed by including the kinase in the whole-cell pipette and measuring EPSC amplitude over time. Synaptic events were recorded in the soma of hippocampal CA1 pyramidal cells with Cs-gluconate-based electrode solutions containing varying
25 concentrations of PKM ζ (0.1, 1, 10 nM). With cells voltage-clamped at the IPSC reversal potential (E_{IPSC} , \sim -70 mV), isolated ensemble EPSCs were elicited by low-frequency (0.1 Hz) electrical stimulation applied in stratum radiatum. Stimulus intensity was set at moderate
30 levels (\sim 5 V) to selectively evoke fast EPSCs (i.e., AMPA/kainate-mediated) and was held constant throughout the experiment. Control data consisted of measurements

of EPSC amplitude and duration immediately following establishment of a whole-cell recording (i.e., before PKM ζ diffusion into the target cell), and parallel experiments with the kinase denatured by boiling or
5 inactivated by multiple freeze/thaw. Activity of the perfused PKM ζ was determined by phosphorylation assay in vitro with each experiment.

EPSCs was continuously monitored to assess the effects of intracellular PKM ζ on peak EPSC magnitude and
10 duration. Periodically, current-voltage records were taken to check constancy of the EPSC reversal potential (E_{EPSC}), and the input resistance of the cell was monitored throughout the experiment. Application of the non-NMDA-receptor antagonist CNQX (10 μ M) was applied to slices to
15 confirm that EPSCs elicited at E_{IPSC} with moderate stimulus intensity were mediated solely through non-NMDA receptors.

Once an effect was observed and appeared stable, the competitive PKC catalytic domain inhibitor,
20 chelerythrine, was added to the bath to attempt to reverse the effect by preventing PKM ζ 's phosphorylation. The drug was then washed out. An example of an experiment with chelerythrine using 3 nM PKM ζ is provided in Figures 2A-2B.

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EXAMPLE 3

Purification of baculovirus-expressed PKM ζ from Sf9 cells: Spodoptera frugiperda (Sf9) cells were grown in SF-900 II SFM insect cell culture medium (Gibco) containing 5 μ g/ml gentamicin. To express the PKM ζ , 5
10x10⁸ cells of a healthy, log phase Sf-9 culture were spun, resuspended in 125 ml of medium, and infected with 25 ml of the baculovirus- ζ virus stock (gift of Sylvia Stable). Following a 0.5 hr incubation at room
10 temperature, additional medium was added to the cells, which were then seeded at a density of 1x10⁶ cells/ml. After 3 days, the cells were spun, washed with PBS, and then homogenized in 65 ml of homogenization buffer. A 2-
15 step purification, employing DEAE Fast Flow Sepharose and Superdex 75 (preparation grade, Pharmacia) columns, was performed to purify baculovirus-expressed PKM ζ . Activity of the PKM ζ was assayed on the same day as each whole cell experiment. Silver stain of baculovirus, recombinantly expressed PKM ζ showing purity of
20 preparation. (See Fig. 3)

EXAMPLE 4Specificity of chelerythrine as inhibitor of PKM ζ

5 Inhibition by the drug chelerythrine of phosphorylation of exogenous substrates by various protein kinases showed that chelerythrine was greater than 10-fold selective as an inhibitor of PKM ζ activity. (See Figure 4).

EXAMPLE 5**Sense, antisense, and amino-acid sequence of human PKM ζ**

Human sequence, obtained from published
expressed sequence tags, were obtained by analogy with
5 rat sequence (Ono, et al., 1988), which was identified as
containing the open reading frame of PKM ζ . The PKM ζ
sequence is used for gene transfer technology to increase
levels of PKM ζ in the nervous system. Dominant negative
inhibitors of PKM ζ are obtained by changing or deleting
10 sequences in the ATP-binding site or by administering
selective domains of the protein, e.g., the carboxy-
terminal domain.

Dominant negative PKM ζ was obtained by
elimination or alteration of ATP-binding domain (marked),
15 and by administration of carboxy-terminal domain
(marked). Antisense was achieved by administration of
all or part of antisense sequence to PKM ζ . (See Figure
6).

EXAMPLE 6

In order to confirm that PKM ζ mediates potentiation of synaptic transmission during LTP, a dominant negative inhibitory form of PKM ζ (PKM ζ -K281W) was introduced postsynaptically into a CA1 pyramidal cell its effect on LTP was examined. The lysine²⁸¹ to tryptophan mutation in the catalytic domain of PKM ζ -K281W) abolished kinase activity by disrupting ATP-binding. Twenty nanomolar of the dominant negative inhibitor (Fig. 7A) was included in a whole-cell recording pipette and allowed to diffuse for 10 min into a CA1 pyramidal cell, voltage clamped at -75 mV to isolated synaptic AMPA responses. PKM ζ -K281W had no obvious effect on synaptic transmission mediated by AMPA and NMDA receptors, sampled at -40 mV at 1 min and 10 min (Fig. 7B). PKM ζ -K281W completely eliminated persistent synaptic potentiation in the cell, but not post-tetanic potentiation (PTP) (Fig. 7C, upper traces, D, large graph). Twenty minutes after the tetanic stimulation, the EPSCs were 101.6 \pm 1.9% of baseline responses (n=4). Simultaneous LTP in the field responses of the slice was observed (Fig. 7C, lower traces, and D, inset graph).

PKM ζ -K281W was expressed using the MaxBac 2.0 Baculovirus/Sf9 system (Invitrogen, Carlsbad, CA). An insert containing amino acids 158-592 of PKC ζ (Drier, et al. (2000) 30th Annual Meeting of the Society of Neuroscience, New Orleans, LA, generous gift from Jerry Yin, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was subcloned into the baculovirus transfer vector pBlueBacHis2B between the EcoRI and SalI sites. The construct was sequenced for verification, and then cotransfected into Sf9 cells with linearized baculovirus

genome. Recombinant protein was expressed in Sf9 cells, and purified using a Ni^{2+} column (Invitrogen, Xpress™ Purification Kit, Carlsbad, CA), and analyzed by silver stain (Fig. 7A) and Western blot (data not shown).

WHAT IS CLAIMED IS:

1. A method of enhancing memory in an animal comprising the administration of a therapeutically effective amount of an atypical form of PKC.

5 2. The method of claim 1 wherein said atypical form of PKC is administered in an amount resulting in final concentrations of said atypical form of PKC in brain ranging from about 0.1 to about 10 nanomolar.

10 3. A method of enhancing synaptic transmission in an animal comprising the administration of a therapeutically effective amount of an atypical form of PKC.

15 4. A method of maintaining memory in an animal comprising the administration of a therapeutically effective amount of an atypical form of PKC.

 5. A method of treating brain injury in an animal comprising the administration of a therapeutically effective amount of an atypical form of PKC.

20 6. A method of treating spinal cord injury in an animal comprising the administration of a therapeutically effective amount of an atypical form of PKC.

 7. The method of any one of Claims 1, 3, 4, 5 or 6 where said atypical form of PKC is PKM ζ .

25 8. The method of any one of Claims 1, 3, 4, 5 or 6 wherein said atypical form of PKC is PKC ι/λ .

30 9. A pharmaceutical composition comprising an atypical form of PKC and a pharmaceutically acceptable carrier.

10. The composition of Claim 9, wherein said atypical form of PKC is PKM ζ or PKC iota/lambda.

5 11. A method of causing amnesia or decreasing synaptic transmission in an animal suffering from a traumatic stress disorder, a phobia, a pain syndrome or epilepsy comprising the administration of a therapeutically effective amount of a PKM ζ inhibitor.

12. The method of claim 11 wherein said PKM ζ inhibitor is chelerythrine.

10 13. The method of Claim 8 wherein said PKM ζ inhibitor is myristolated zeta inhibitory pseudosubstrate peptide..

15 14. The method of claim 12, wherein said inhibitor is a dominant negative or altered form of PKM ζ , or antisense version of PKM ζ .

15 15. The method of any one of claims 1, 3-6 and 11 wherein said animal is a human.

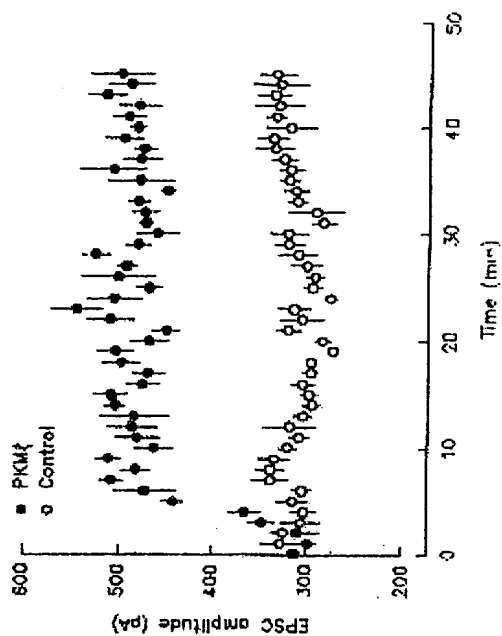


Figure 1B

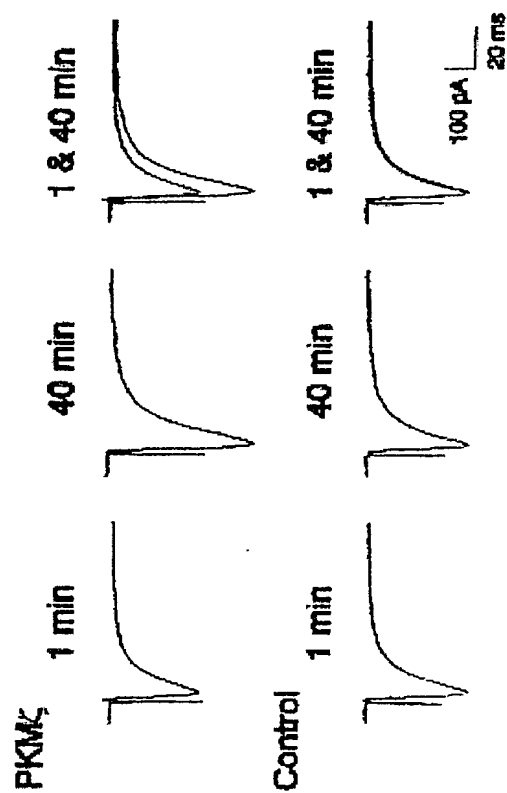


Figure 1A

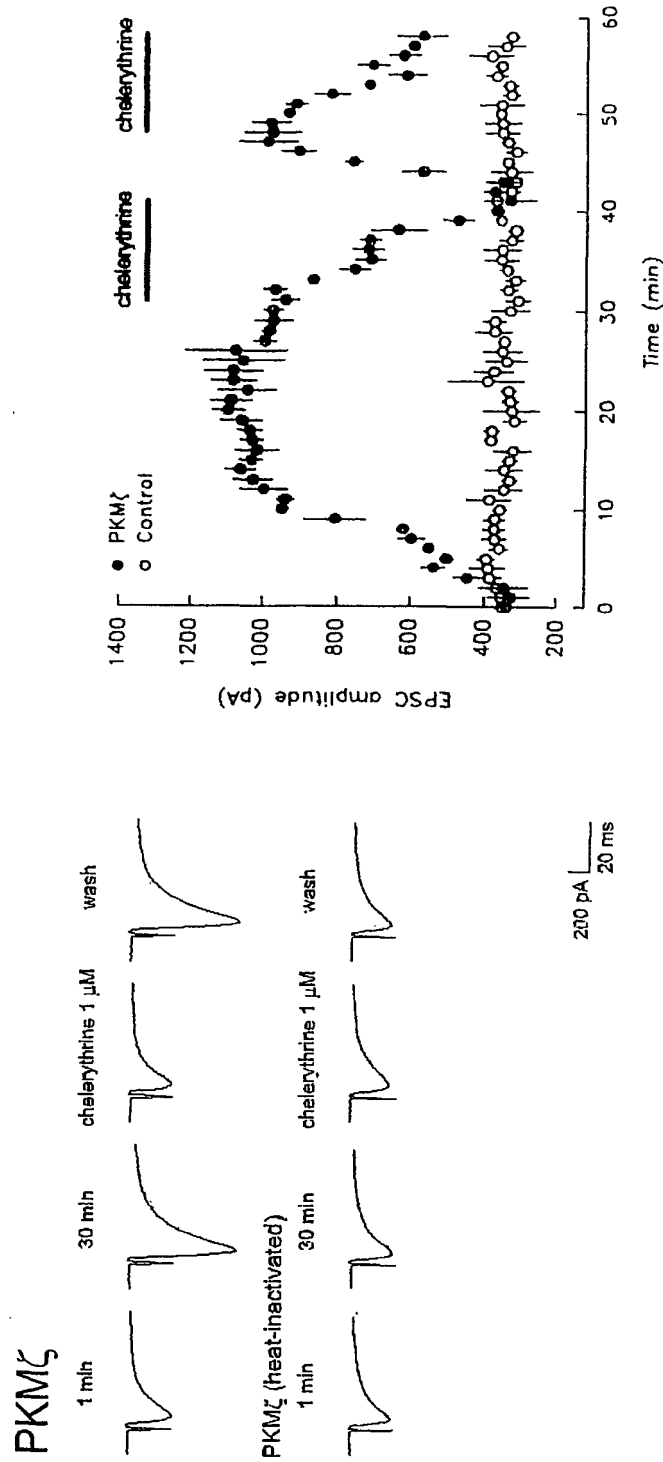


Figure 2B

Figure 2A

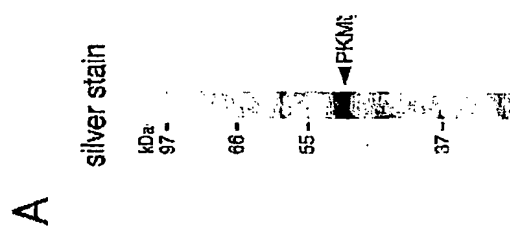


Figure 3

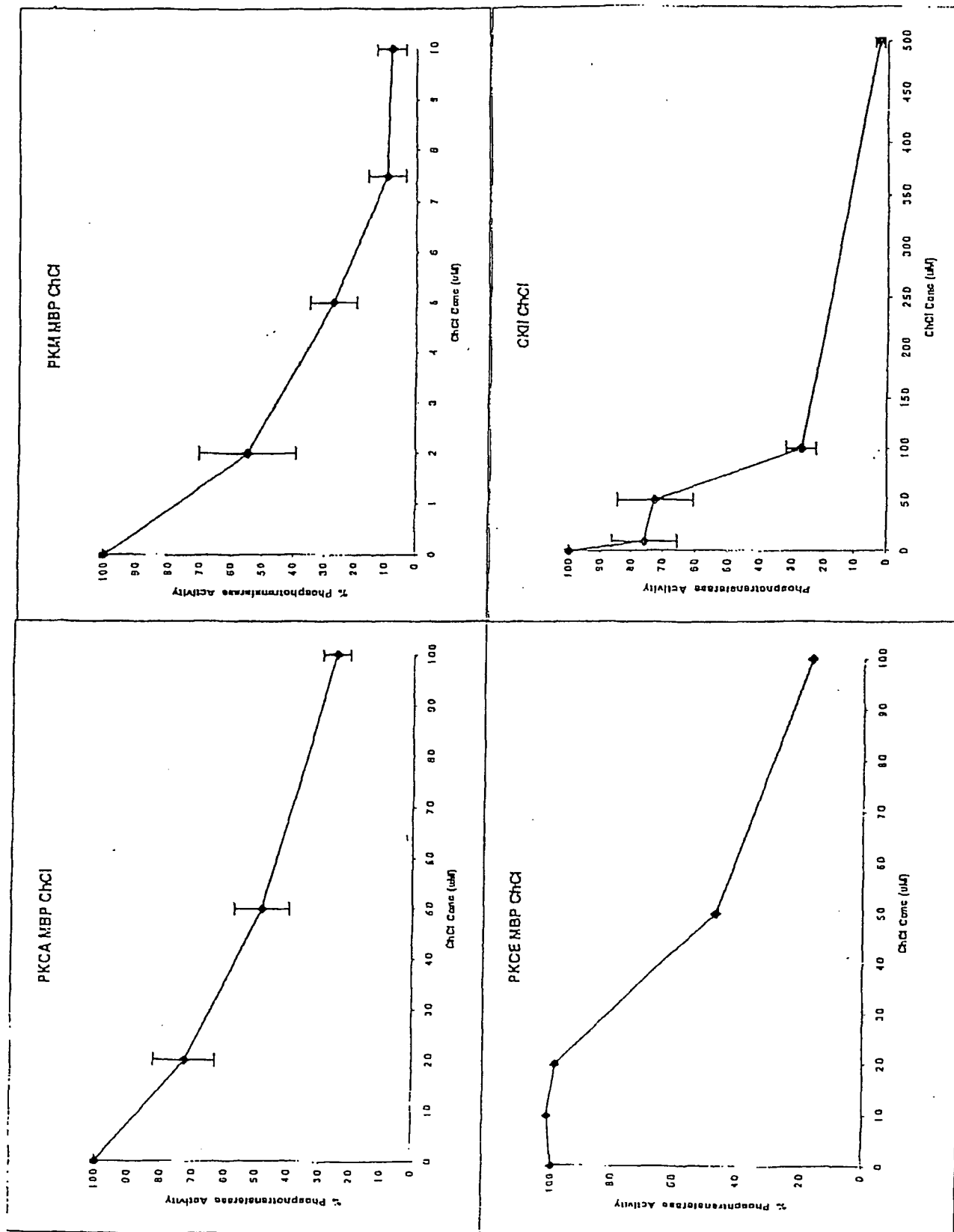


FIGURE 4

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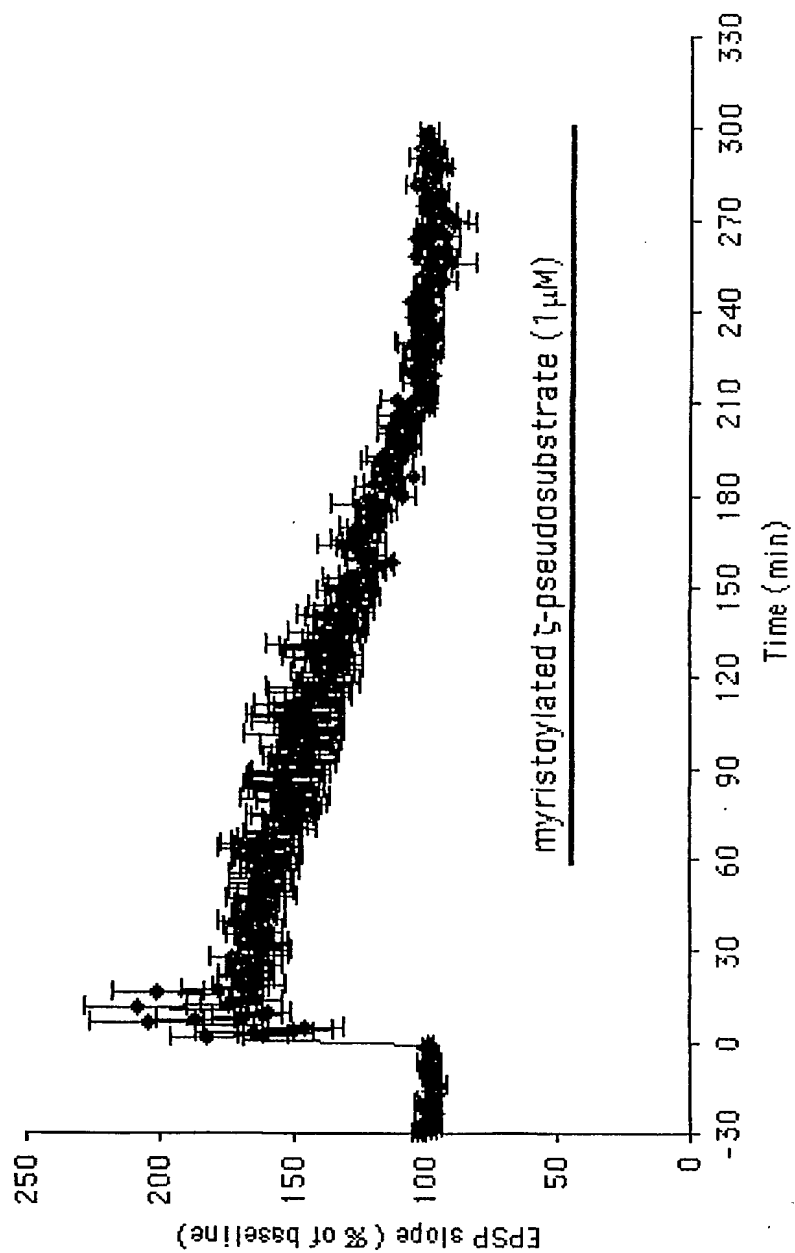


Figure 5

Sense : C C C G G G C C T G G A G A C A T G A G G A G G C A G G G A T G T G A G G G G C G G G G A C A G G
 Antisense: G G G C C C G G A C C T C T G T A C T C C T C C G T C C C T A C A C T C C C C G C C C C C T G T C C

60 70 80 90 100
 A C A G C C G G C C T T C C G T T A A A T A T C T G C T C C T C G C G C T C G A G C C T C C C T G C
 T G T C G G C C G G A A G G C A T T T A T A G A C G A G G A G C G C G A G C T C G G A G G G A C G

110 120 130 140 150
 C T A T T G T C G G G G C C G A G C G A A G C C G A C G C A G C A T C A G C T C G T C A A C G G G
 G A T A A C A G C C C C G G C C T C G C T T C G G C T G C G T C G T A G T C G A G C A G T T G C C C

160 170 180 190 200
 A A G G A A G A T G C C T C C C T G C A C G C C C G C C G C G C A C A G A G C A T A A A G A A T C T
 T T C C T T C T A C G G A G G G A C G T G C G G C G G C G C G T G T C T C G T A T T T C T T A G A

210 220 230 240 250
 G C G C T G A G G A G G C A G G A G A A A G C C G A A T C T A T C T A C C G C C G G G G A G C
 C G C G A C T C C T C C G T C C T C T T C T T C G G C T T A G A T A G A T G G C G G C C C C T C G

260 270 280 290 300
 C A G A A G A T G G A G G A A G C T G T A C C G T G C C A A C G G C C A C C T C T T C C A A G C C A
 G T C T T C T A C C T C C T T C G A C A T G G C A C G G T T G C C G G T G G A G A A G G T T C G G T

310 320 330 340 350
 A G C C C T T T A A C A G G A G A G C G T A C T G C G G T C A G T G C A G C G A G A G G A T A T N G
 T C G C G A A A T T G T C C T C T C G C A T G A C G C C A G T C A C G T C G C T C T C C T A T A N C

360 370 380 390 400
 G G C C T C G C G A G G C A A G G C T A C A G G T G C A T C A A C T G C A A A C T G C T G G T C C A
 C C G G A G C C C T C C G T T C C G A T G T C C A C G T A G T T G A C G T T T G A C G A C C A G G T

410 420 430 440 450
 T A A G C G C T G C C A C G G C C T C G T C C C G C T G A C C T G C A G G A A G C A T A T G G A T T
 A T T C G C G A C G G T G C C G G A G C A G G G C G A C T G G A C G T C C T T C G T A T A C C T A A

Protein: M D>

460 470 480 490 500
 C T G T C A T G C C T T C C C A A G A G C C T C C A G T A G A C G A C A A G A A C G A G G A C G C C
 G A C A G T A C G G A A G G G T T C T C G G A G G T C A T C T G C T G T T C T T G C T C C T G C G G
 S V M P S Q E P P V D D K N E D A>

510 520 530 540 550
 G A C C T T C C T T C C G A G G A G A C A R A T G G A A T T G C T T A C A T T T C C T C A T C C C G
 C T G G A A G G A A G G C T C C T C T G T Y T A C C T T A A C G A A T G T A A A G G A G T A G G G C
 D L P S E E T X G I A Y E S S S R>

FIGURE 6

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      560      570      580      590      600
GAAGCATGACAGCATTAAAGACGACTCGGAGGACCTTAAGCCAGTTATCG
CTTCGTACTGTCGTAATTTCTGCTGAGCCTCCTGGAATTCGGTCAATAGC
  K  E  D  S  I  K  D  D  S  E  D  L  K  P  V  I>

      610      620      630      640      650
ATGGGATGGATGGAATCAAAATCTCTCAGGGGCTTGGGGCTGCAGGACTTT
TACCCTACCTACCTTAGTTTTAGAGAGTCCCCGAACCCGACGTCCTGAAA
  D  G  M  D  G  I  K  I  S  Q  G  L  G  L  Q  D  F>

      660      670      680      690      700
GACCTAATCAGAGTCATCGGGCGCGGGAGCTACGCCAAGGTTCTCCTGGT
CTGGATTAGTCTCAGTAGCCCGCGCCCTCGATGCGGTTCCAAGAGGACCA
  D  L  I  R  V  I  G  R  G  S  Y  A  K  V  L  L  V>
      <---ATP-Binding Site----->

      710      720      730      740      750
CGCGTTGAAGAAGAATGACCAAATTTACGCCATGAAAGTGGTGAAGAAAG
CGCCAACTTCTTCTTACTGGTTTAAATGCGGTACTTTCACTACTTCTTTC
  R  L  K  K  N  D  Q  I  Y  A  M  K  V  V  K  K>
      -----ATP-Binding Site----->

      760      770      780      790      800
AGCTGGTGCATGATGACGAGGATATTGACTGGGTACAGACAGAGAAGCAC
TCGACCACGTACTACTGCTCCTATAACTGACCCATGTCTGTCTCTTCGTG
  E  L  V  H  D  D  E  D  I  D  W  V  Q  T  E  K  H>

      810      820      830      840      850
GTGTTTGAGCAGGCATCCAGCAACCCCTTCCTGGTTCGGATTACACTCCTG
CACAAACTCGTCCGTAGGTTCGTTGGGGAAGGACCAGCCTAATGTGAGGAC
  V  F  E  Q  A  S  S  N  P  F  L  V  G  L  H  S  C>

      860      870      880      890      900
CTTCCAGACGACAAGTCGGTTGTTTCCTGGTTCATTGAGTACGTCAACGGCG
GAAGGTCTGCTGTTTCAGCCAACAAGGACCAGTAACTCATGCAGTTGCCGC
  F  Q  T  T  S  R  L  F  L  V  I  E  Y  V  N  G>

      910      920      930      940      950
GGGACCTGATGTTCCACATGCAGAGGCAGAGGAAGCTCCCTGAGGAGCAC
CCCTGGACTACAAGGTGTACGTCTCCGTCTCCTTCGAGGGACTCCTCGTG
  G  D  L  M  F  H  M  Q  R  Q  R  K  L  P  E  E  H>

      960      970      980      990      1000
GCCAGGTTCTACGCGGCCGAGATCTGCATCGCCCTCAACTTCCTGCACGA
CGGTCCAAGATGCGCCGGCTCTAGACGTAGCGGGAGTTGAAGGACGTGCT
  A  R  F  Y  A  A  E  I  C  I  A  L  N  F  L  H  E>

```

FIGURE 6 (CONT'D)

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SUBSTITUTE SHEET (RULE 26)

1010 1020 1030 1040 1050
GAGGGGGATCATCTACAGGGACCTGAAGCTGGACAACGTCCTCCTGGATG
CTCCCCCTAGTAGATGTCCCTGGACTTCGACCTGTTGCAGGAGGACCTAC
R G I I Y R D L K L D N V L L D>

1060 1070 1080 1090 1100
CGGACGGGCACATCAAGCTCACAGACTACGGCATGTGCAAGGAAGGCCTG
GCCTGCCCGTGTAGTTGAGTGTCTGATGCCGTACACGTTCCCTTCCGGAC
A D G E I K L T D Y G M C K E G L>

1110 1120 1130 1140 1150
GGCCCTGGTGACACAACGACCACTTTCTGCGGAACCCCGAATTACATCGC
CCGGGACCACTGTGTGCTCGTGAAAGACGCCTTGGGGCTTAATGTAGCG
G P G D T T S T F C G T P N Y I A>

1160 1170 1180 1190 1200
CCCCGAAATCCTGCGGGGAGAGGAGTACGGGTTTACGCTGGACTGGTGGG
GGGGCTTTAGGACGCCCCCTCTCTCATGCCCAAGTCGCACCTGACCACCC
P E I L R G E E Y G F S V D W W>

1210 1220 1230 1240 1250
CGCTGGGAGTCCTCATGTTTGAGATGATGGCCGGGCGCTCCCCGTTCCGAC
GCGACCCTCAGGAGTACAACTCTACTACCGGCCCGCGAGGGGCAAGCTG
A L G V L M F E M M A G R S P F D>

1260 1270 1280 1290 1300
ATCATCACCGACAACCCCGACATGAACACAGAGGACTACCTTTTCCAAGT
TAGTAGTGGCTGTGGGCCTGTACTTGTGTCTCCTGATGGAAAAGGTTCA
I I T D N P D M N T E D Y L F Q V>

1310 1320 1330 1340 1350
GATCCTGGAGAAGCCCATCCGGATCCCCCGGTTCTGTCCGTCAAAGCCT
CTAGGACCTCTTCGGGTAGGCCTAGGGGGCCAAGGACAGGCAGTTTCGGA
I L E K P I R I P R F L S V K A>

1360 1370 1380 1390 1400
CCCATGTTTTTAAAAGGATTTTTTAAATAAGGACCCCAAAGAGAGGCTCGGC
GGGTACAAAATTTTCCTAAAAATTTATTCCTGGGGTTTCTCTCCGAGCCG
S H V L K G F L N K D P K E R L G>

1410 1420 1430 1440 1450
TGCCGGCCACAGACTGGATTTTCTGACATCAAGTCCCACGCGTTCTTCCG
ACGGCCGGTGTCTGACCTAAAAGACTGTAGTTCAGGGTGCGCAAGAAGGC
C R P Q T G F S D I K S H A F F R>

FIGURE 6 (CONT'D)

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SUBSTITUTE SHEET (RULE 26)

1460 1470 1480 1490 1500
CAGCATAGACTGGGACTTGCTGGAGAAGAAGCAGGCGCTCCCTCCATTCC
GTCGTATCTGACCCTGAACGACCTCTTCTTCGTCCGCGAGGGAGGTAAGG
S I D W D L L E K K Q A L P P F>

1510 1520 1530 1540 1550
AGCCACAGATCACAGACGACTACGGTCTGGACAACTTTGACACACAGTTC
TCGGTGTCTAGTGTCTGCTGATGCCAGACCTGTTGAAACTGTGTGTCAAG
Q P Q I T D D Y G L D N F D T, Q F>

1560 1570 1580 1590 1600
ACCAGCGAGCCCGTGCAGCTGACCCAGACGATGAGGATGCCATAAAGAG
TGGTCGCTCGGGCACGTCGACTGGGGTCTGCTACTCCTACGGTATTTCTC
T S E P V Q L T P D D E D A I K R>

1610 1620 1630 1640 1650
GATCGACCAGTCAGAGTTCCAAGGCTTTGAGTATATCAACCCATTATTGC
CTAGCTGGTCAGTCTCAAGCTTCCGAAACTCATATAGTTGGGTAATAACG
I D Q S E F E G F E Y I N P L L>

1660 1670 1680 1690 1700
TGTCCACCGAGGAGTCGGTGTGAGGCCGCGTCCGTCTCTGTCTGGACAC
ACAGGTGGCTCCTCAGCCACACTCCGGCGCACGCAGAGACAGCACCTGTG
L S T E E S V>
----- C-terminus --->

1710 1720 1730 1740 1750
GCGTGATTGACCCTTTAACTGTATCCTTAACCACCGCATATGCATGCCAG
CGCACTAACTGGGAAATTGACATAGGAATTGGTGGCGTATACGTACCGTC

1760 1770 1780 1790 1800
GCTGGGCACGGCTCCGAGGGCGGCCAGGGACAGACGCTTGCGCCGAGACC
CGACCCGTGCCGAGGCTCCCGCCGGTCCCTGTCTCGAACGCGGCTCTGG

1810 1820 1830 1840 1850
GCAGAGGGAAGCGTCAGCGGGCGCTGCTGGGAGCAGAACAGTCCCTCACA
CGTCTCCCTTCGCAGTCGCCCCGCGACGACCTCGTCTTGTCAGGGAGTGT

1860 1870 1880 1890 1900
CCTGGCCCCGGCAGGCAGCTTCGTGCTGGAGGAAGTTGCTGCTGTGCCTGC
GGACCGGGCCGTCCGTGCAAGCACGACCTCCTTGAACGACGACACGGACG

1910 1920 1930 1940 1950
GTCGCGGCGGATCCGCGGGGACCTTGCCGAGGGGGCTGTCATGCGGTTTC
CAGCGCCGCGCTAGGCGCCCCCTGGGACGGCTCCCCCGACAGTACGCCAAAG

FIGURE 6 (CONT'D)

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SUBSTITUTE SHEET (RULE 26)

1960	1970	1980	1990	2000
CAAGGTGCACATTTTCCACGGAAACAGAACTCGATGCACTGACCTGCTCC				
GTTCCACGTGTAAAAGGTGCCTTTGTCTTGAGCTACGTGACTGGACGAGG				

2010	2020	2030	2040	2050
GCCAGGAAAGTGAGCGTGTAGCGTCCTGAGGAATAAAATGTTCCGATGAA				
CGGTCCTTTCACCTCGCACATCGCAGGACTCCTTATTTTACAAGGCTACTT				

AAAAAAAAA
TTTTTTTTT

FIGURE 6 (CONT'D)

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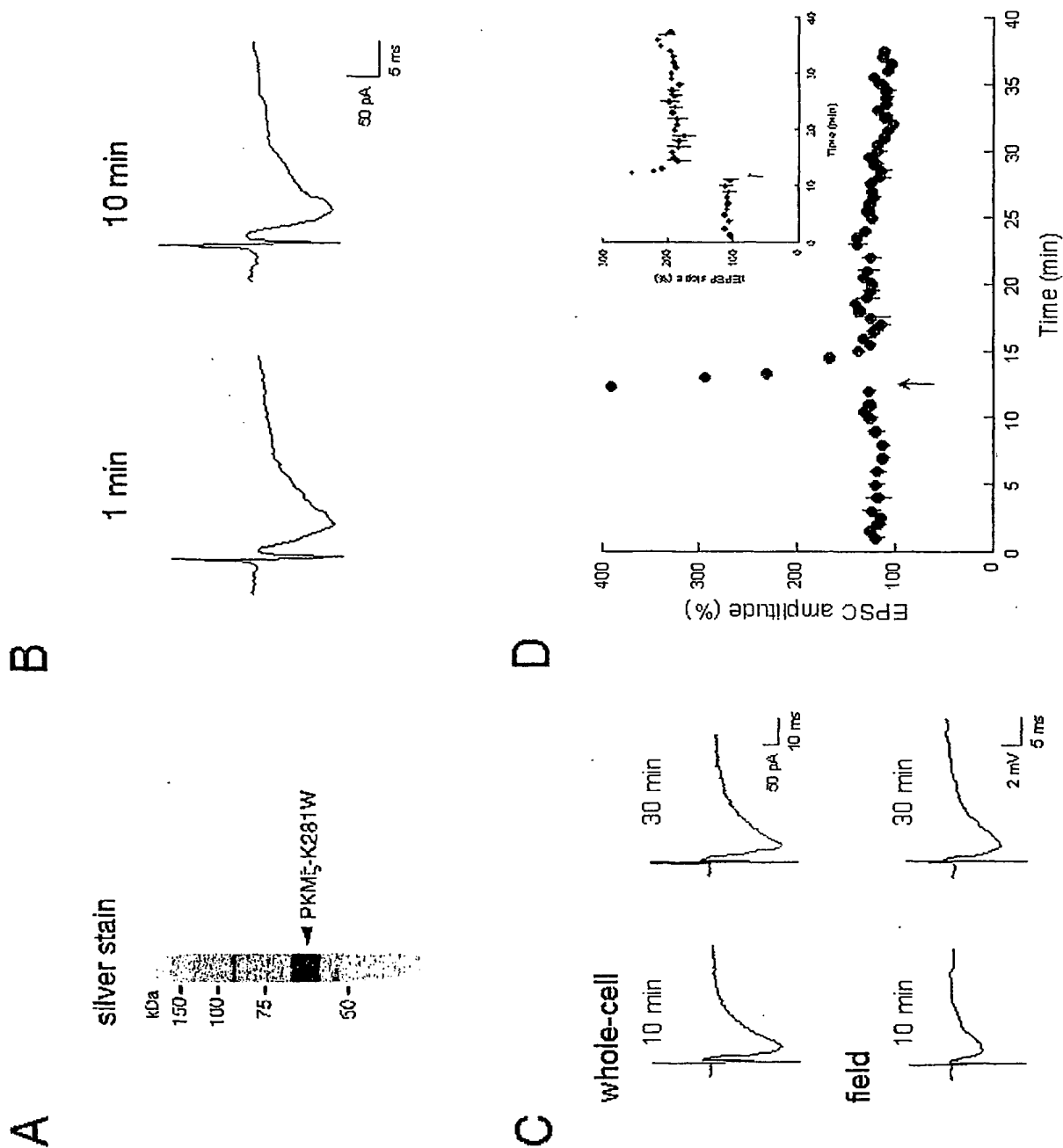


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/12794

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/16, 38/17, 38/43, 38/45

US CL :435/193; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/193; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SACKTOR et al. Persistent activation of the ζ isoform of protein kinase C in the maintenance of long-term potentiation. 15 September 1993, Vol. 90, No. 18, pages 8342-8346, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 JUNE 2001

Date of mailing of the international search report

26 JUL 2001

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Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MICHAEL PAK

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/12794

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BRS, MEDLINE

search terms: protein kinase C, PKC, PKM, memory, inject, administration

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